

# Nitrate Removal from Groundwater Using Immobilized Heterotrophic Algae

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## Abstract

The treatment efficiency of *Chlorella sorokiniana* and *Scenedesmus species*, immobilized in sodium alginate, was evaluated for removing nitrate from groundwater. The experiments were performed initially in batch mode and the best-performing conditions were replicated in sequencing batch reactor mode. *S. sp.* showed a higher nitrate uptake in short term than *C. sorokiniana*. Immobilized *S. sp.* and *C. sorokiniana* cells showed 90% nitrate removal in 9 and 12 days, respectively. The optimal ratio of algal beads/water was found to be 12.5% (v:v). Comparatively, suspended *S. sp.* cells were able to remove only up to 35% of nitrate in 8 days. Alginate immobilized *S. sp.* beads were capable of uptaking nitrate for 100 consecutive days in sequencing batch reactor mode. When tested in actual groundwater, 90% of nitrate was eliminated in 2 days without need for any additional carbon source. Immobilized algal beads can be a low-cost alternative technique to remove nitrate from groundwater as they are water-insoluble, non-toxic, easy to harvest, and offer high removal efficiency.

**Keywords:** *Scenedesmus species*, *Chlorella sorokiniana*, Biological nitrate treatment, Nitrate removal, nitrogen, heterotrophic growth

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**Supplementary material** follows the **References**.

**Highlights:** Utilization of immobilized algae is a practical method for nitrate treatment. • Cultivation of heterotrophic algae reduces retention time needed for treatment. • Actual groundwater is more suitable for nitrate removal than simulated lab water. • In natural water, additional carbon source is not required for successful removal. • Same algae beads could be reused for treatment for repeated cycles up to 100 days.

## 1 Introduction

Nitrate is the most common contaminant in the groundwater's aquifers (Wheeler et al. 2015). About 21% of public water supply and 98% of self-supplied water in 2010 was extracted from groundwater (Survey 2010). The extensive use of nitrate based fertilizers is the largest contributor to nitrate contamination; therefore, the highest concentration of nitrate in groundwater has been observed in agricultural areas (Lawniczak et al. 2016). US Environmental Protection Agency (US EPA) has set a maximum contaminant level (MCL) of 10 mg/l  $\text{NO}_3\text{-N}$  for nitrate. (Agency 2018). Exposure to drinking water with a nitrate level above the MCL is a potential health risk for infants and sometimes, for adults. Methemoglobinemia which is the result of nitrate reduction to nitrite in infants' digestive system is reported to be common between infants who have consumed water with elevated levels of nitrate (Liu et al. 2012). Moreover, exposure to drinking water contaminated with nitrate and atrazine, a type of herbicide used as weed killer, is reported as a potential health risk, because it can form nitrosamine. Nitrosamine can increase the risk of developing non-Hodgkin lymphoma (Rhoades et al. 2013).

Reverse osmosis (RO) and ion exchange are generally considered to be the best available nitrate treatment technologies; however, they are associated with high capital and operating costs. Furthermore, RO membranes retain nitrate and other contaminants into a concentrated waste brine that poses a disposal problem (Zhang et al. 2012b). Most available ion exchange nitrate removal resins suffer from the inherent natural low selectivity for  $\text{NO}_3$ . Most anionic resins are more selective for  $\text{SO}_4$  (than  $\text{NO}_3$ ) which is also a common groundwater constituent, usually in higher concentrations than  $\text{NO}_3$ . Therefore, there is a need to find an economical, sustainable water treatment technique which can diminish the nitrate concentration.

Microalgae treatment is a sustainable and effective approach for the removal of nitrate from groundwater. Algae is capable of up-taking nitrate and creating biomass that concentrates the nitrogen. Strains of *Chlorella vulgaris* and *Scenedesmus sp.* have already shown promising results in nitrate removal as immobilized and free cells in wastewater (AlMomani and Örmeci 2016). However, designing an effective algae photobioreactor is challenging because of the biomass loss in a continuous flow reactor and the difficulty in harvesting algal biomass at the end of treatment process. Algal biomass harvesting requires expensive centrifuging and filtration operations (Moreno-Garrido 2013). Thus, the immobilization approach of microalgae into polymeric matrices will help solve both problems. Immobilized algal cells occupy smaller space and are easier to handle. The immobilization of algae facilitates the harvesting of biomass and protects the cells from the harsh environments (e.g., metal toxicity, pH and salinity) (Eroglu et al. 2012).

Both synthetic and natural polymers can be used as immobilization agents (Moreno-Garrido 2008). Alginate is the most widely used polymer for immobilization of microalgae, as it is a non-toxic, transparent polymer. Transparency of the alginate matrix allows light transmission which is a critical factor for photosynthesis in autotrophic cells (Seli-moglu and Elibol 2010). Several studies have used immobilized algal cells to remove nutrients from wastewater (Ruiz-Marin et al. 2010; Liu et al. 2012). The strains of *Scenedesmus obliquus* and *Chlorella vulgaris*, immobilized in sodium alginate, were used to remove nutrients from wastewater in batch and semi-continuous modes (Ruiz-Marin et al. 2010). *S. obliquus* was reported as a better candidate as it showed higher N and P uptake than *C. vulgaris*. Additionally, *S. obliquus* was more effective in removing N and P for longer periods in semi-continuous mode than in batch cultures.

*Chlorella vulgaris* and *Chlamydomonas sp.*, immobilized in calcium alginate, were used during the tertiary treatment of municipal wastewater. *C. vulgaris* was reported as the best specie with 72% and 99% of reduction in nitrate and orthophosphate, respectively (Shaker et al. 2015). *Chlorella sp.* immobilized in calcium alginate sheets removed 100% of  $\text{NH}_4\text{-N}$  and P from real domestic secondary effluents (Zhang et al. 2012a).

Depending on the algae species used, there are three main cultivation modes which can be applied for immobilized algal beads: photo-autotrophic, heterotrophic, and mixotrophic growth (J. Wang et al. 2014). Photo-autotrophic microalgae use dissolved carbon dioxide and absorb light to perform photosynthesis. In contrast, heterotrophic growth occurs when microalgae perform photosynthesis and use organic carbon as carbon and energy sources without the need of light (Perez- Garcia and Bashan 2015). Mixotrophic cultivation uses both inorganic and organic carbon sources in the presence of light (Abreu et al. 2012). In autotrophic growth, light penetration is inversely proportional to the cell concentration. Therefore, it is more difficult to reach a high density of microalgae biomass using autotrophic growth. As the cells achieve higher density, it gets harder for the light to penetrate into the cells. The low biomass concentration in autotrophic growth also increases the harvesting cost at the end of the treatment process (Liang et al. 2009). In large scale applications, heterotrophic cultivation is cheaper and easier to handle than autotrophic cultivation as it does not have the limitation of light dependency for growth (O. Perez- Garcia et al. 2011). The microalgae species that undergo heterotrophic growth can grow in light-limited conditions and this can lead to the higher biomass production. Strains of *C. sorokiniana* and *S. sp.* can produce biomass under autotrophic, mixotrophic, and heterotrophic conditions (Rai and Gupta 2016; DiCaprio et al. 2018; Kim et al. 2013). Monosaccharides or organic acids can be used as carbon and energy sources in heterotrophic cultivation (Figueroa-Martinez et al. 2015). While wastewater treatment using immobilized algae was previously reported, there are no reported studies on heterotrophic growth of immobilized microalgae to treat nitrate-contaminated groundwater. In this work, both batch and sequencing batch growth studies were conducted to evaluate the treatment efficiency of *C. sorokiniana* and *S. sp.* immobilized in sodium alginate for removing nitrate from groundwater while they undergo autotrophic and heterotrophic growth.

## 2 Materials and Methods

### 2.1 Cultivation of Algae and Preparation of Immobilized Algal Beads

Stock cultures of *C. sorokiniana* and *S. sp.* were obtained from the Biochemistry Department at the University of Nebraska-Lincoln. The composition of the nutrient solution is provided elsewhere (Soriat et al. 1997). The nutrient solution consisted of essential inorganic salts and vitamins necessary to grow micro-organisms:  $B^{+3}$ ,  $Ca^{+2}$ ,  $Cl^{-}$ ,  $Co^{+2}$ ,  $CU^{+2}$ ,  $Fe^{+3}$ ,  $K^{+}$ ,  $Mg^{+2}$ ,  $Mn^{+2}$ ,  $Mo^{+6}$ ,  $NH_4^{-}$ ,  $Na^{+}$ ,  $SO_4^{-2}$ ,  $Zn^{+2}$ , p-aminobenzoic acid, biotin, cyanocobalamin, folic acid, nicotinic acid, pantothenic acid, pyriodoxine hydrochloride, riboflavin, thiamin hydrochloride, and thioctic acid concentration. In addition, a nutrient spike solution (0.29 mM  $NaNO_3$  and 0.03 mM  $NaH_2PO_4 \cdot H_2O$ ) was added to the feed solution to achieve the target of 50 mg/L nitrate. The algal solution was kept at room temperature. Both artificial plant light and sunlight were used for algal growth and the intensity of light was ranged between 500 and 700 lux.

Microalgae were cultivated in a 3-gal container using bulk media solution. After approximately 2 weeks of cultivation, the algae were ready to be used for bead preparation, when the turbidity reached 30 NTU. To prepare alginate-algae beads, algae solution was centrifuged at 3500 rpm for 10 min. The supernatant was discarded, and the algal cells were resuspended in DI water to obtain a concentrated solution. All the growth medium nutrients used to cultivate microalgae, except nitrate, were added to the algae concentrated suspension. Next, the concentrated algae solution was mixed with 1.5% sodium alginate solution. The mixture was dropped into a 2% calcium chloride solution using a syringe pump (Harvard Apparatus, model 55-3333) and left overnight to form algal beads. The algal beads were uniform and had the diameter of 2.6 mm. The beads were rinsed and kept in DI water before being used for the experiments.

The water matrix used in all batch and sequencing batch experiments—except the one with actual groundwater—was DI water, containing 10 mg/L  $NO_3$ -N. Potassium nitrate (99.9%, Sigma-Aldrich) was used and the concentration of  $NO_3$ -N in all reactors was measured using UV-vis spectrophotometer (Spectronic, model Genesys 5). The actual groundwater sample used at the final stage of the experiments

was collected from a pump station at city of Hastings, NE. The concentration of dissolved oxygen (DO) in each reactor was measured on daily basis, using DO meter (YSI, model 5100). A pH meter (Thermo Scientific, model Orion 3-star) and a conductivity meter (Hach, model HQ14D) were used to record pH and conductivity daily. The COD of the bulk solution was measured using TNT-plus vial tests (Hach, TNT820). GE plant light kits were used as light sources. The intensity of light was measured using a digital light meter (Leaton, model 935976) and maintained between 780 and 1000 lux.

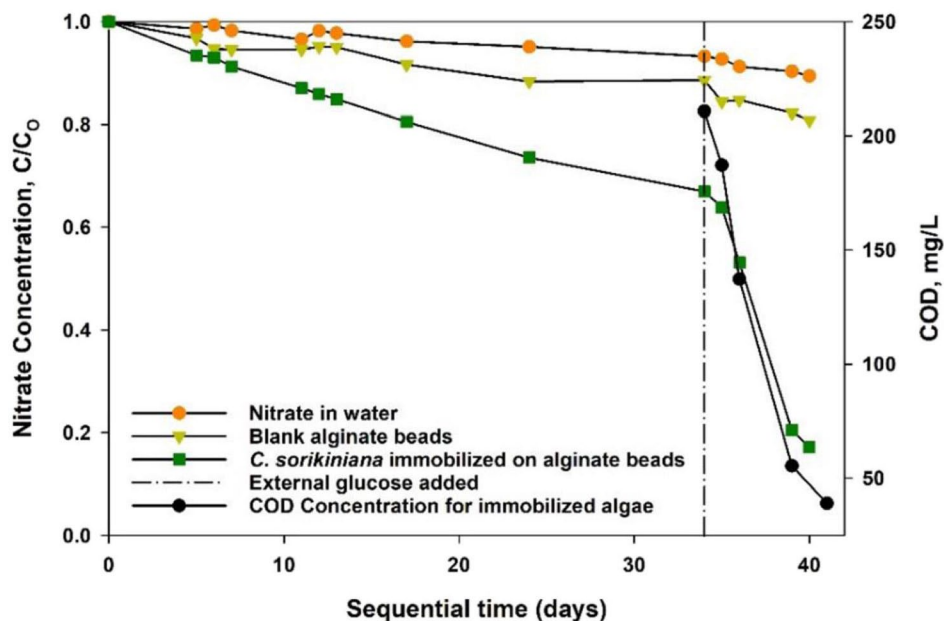
## **2.2 Viability and Growth Assessment**

An assessment of the viability of algal cells within the beads was performed by observation of algal colonies under Ti-S inverted fluorescence microscope (Nikon, Melville, NY). The chloroplast in algae contains chlorophylls, which are light-harvesting molecules, embedded in the thylakoid membrane. The emission spectrum of chloroplasts shows strong autofluorescence in red, with a peak at 680 nm (Kodama 2016). The microscopic images were used to isolate the live algal cells from the background using the color threshold function in ImageJ v1.51 j8. The total area covered in red was recorded as population of live cells.

Detailed images of the beads were taken using scanning electron microscopy (SEM). SEM images were acquired using a Nova Nano SEM 450 (FEI, Hillsboro, OR), with the voltage ranging between 2 and 5 kV. The air-dried algal beads were coated with a thin layer of gold before imaging.

The dry cell weight (DCW) measurements were performed in sequencing batch reactors to determine the density and growth rate of algal cells inside of the beads. Five beads from each reactor were dissolved in 5 ml of 4% sodium bicarbonate solution. The suspension was then filtered through a 3- $\mu$ m (pore size) filter paper, leaving a layer of microalgae on the paper. The dry weight of the microalgae was measured after drying the filter paper at 100 °C for 24 h.

All batch and sequencing batch studies were performed in 500 mL flasks, containing 400 mL DI water and 10 mg/L  $\text{NO}_3\text{-N}$ . All required macro- and micronutrients were supplied imbedded in the beads. Glucose, when needed, was added at a concentration of 125 mg/ L. For the samples with actual groundwater, no additional nutrients or glucose was provided in the bulk solution.



**Fig. 1** Percentage of nitrate present (left vertical axis) and change in concentration of COD (right vertical axis) of the solution over time. At the end of day 34, 50 mg glucose was added to each reactor.

### 3 Results and Discussion

#### 3.1 Impact of Autotrophic Vs. Heterotrophic Conditions on Nitrate Removal

To simulate the autotrophic and heterotrophic growth of algae, batch studies were performed with and without addition of an organic carbon source. At autotrophic condition, 75 mL of *C. sorokiniana* beads were run for 34 days without adding any organic carbon source. The dissolved atmospheric CO<sub>2</sub> (~ 1.5 g/L) was the only source of carbon. Two types of controls were operated simultaneously: one reactor with alginate beads (no microalgae) and another reactor containing only deionized water. At day 34, glucose (125 mg/L) was added to the bulk solution to facilitate heterotrophic growth. In the first 5 days, immobilized *C. sorokiniana* beads showed 6.5% nitrate removal which reached 33% after 34 days of autotrophic growth. As shown in **Fig. 1**, the removal efficiency increased to 36%, only 1 day after adding glucose to the bulk (day 35) and reached to 82%, at day 40. Since glucose is the only source of organic carbon in the bulk, the COD



concentration was used to measure the glucose consumption by algal cells. The change in concentration of COD in the solution indicates its complete consumption by algal cells.

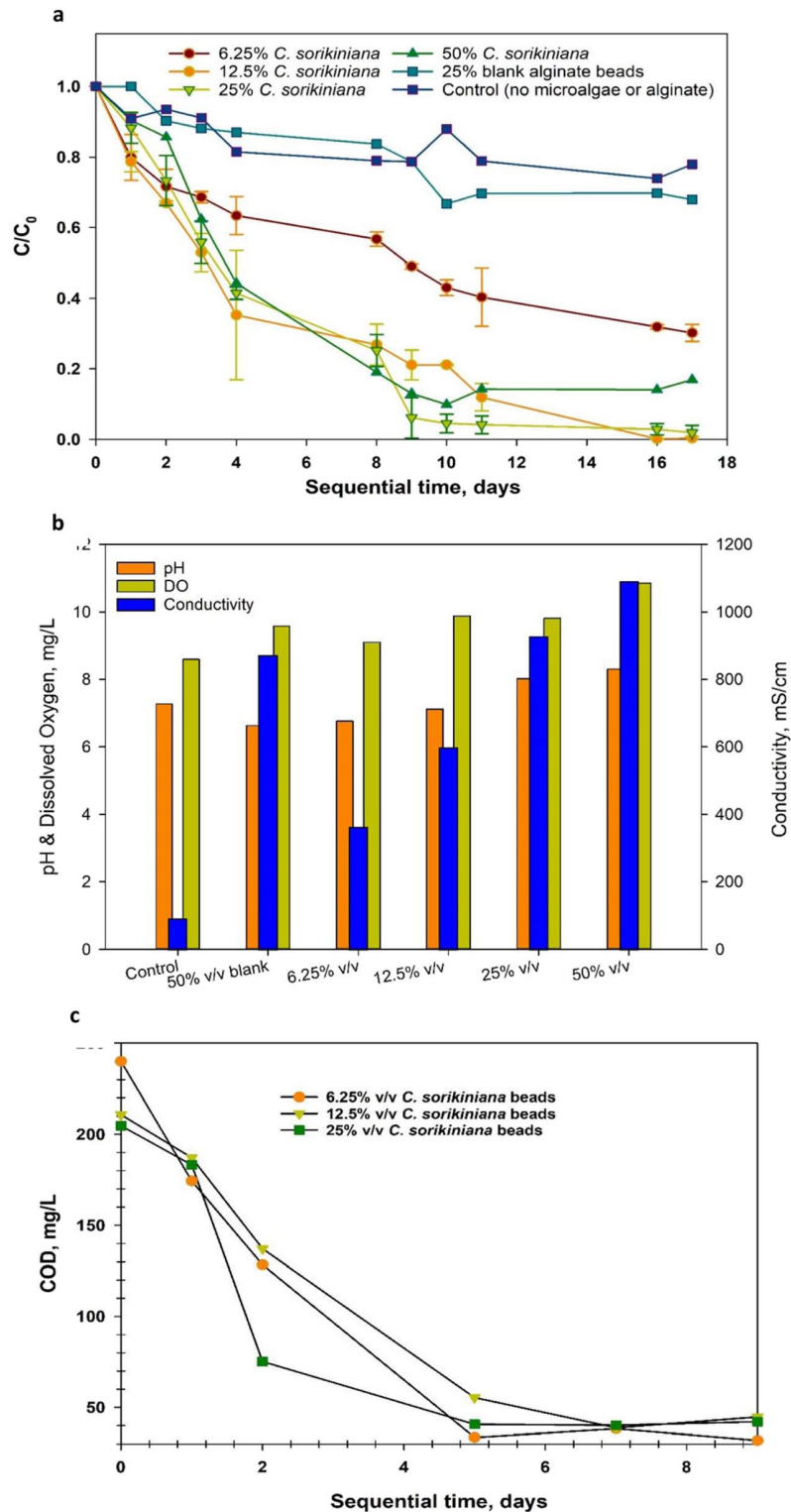
The effect of light on the heterotrophic growth of algal beads was also evaluated by measuring the nitrate uptake rate and DCW in complete darkness, medium (300 lux) and high (700 lux) intensity light. The highest DCW and nitrate uptake was observed in presence of high intensity light source.

Studies on microalgae suggest that only two enzymes, nitrate reductase (NR; EC 1.6.6.1-3) and nitrite reductase (NiR; EC 1.7.7.1), are responsible to catalyze nitrate to ammonium (Fernandez and Galvan 2007). Different environmental variables affect nitrate assimilation. One of the key factors in nitrate assimilation is the presence of light, as most algae assimilate nitrate faster in the light than in the dark. Light also has an important effect in glucose uptake by microalgae cells. For *Chlorella* cells growing at the presence of glucose, the blue end of the visible spectrum controls many of the metabolic reactions (Karlander and Krauss 1966). The blue light inhibits uptake of glycine, proline, and arginine, but activates the nitrate reductant enzymes and therefore, enhances uptake of oxygen and nitrate by microalgae (Perez-Garcia et al. 2011).

### 3.2 Optimum Volume of Algal Beads Needed

To determine the optimum algal beads/water ratio that maximizes the nitrate removal rate, four different ratios of *C. sorokiniana* beads to water (50%, 25%, 12.5%, and 6.25%) by volume were evaluated. A sample containing 50% blank alginate beads was prepared for control treatments. As shown in **Fig. 2a**, the removal of  $\text{NO}_3\text{-N}$  after 16 days in both control treatments, with no beads and with blank alginate beads, was 28% and 31%, respectively. All other bioreactors with microalgae achieved higher removal than the control. The average of 50%  $\text{NO}_3\text{-N}$  was removed by immobilized *C. sorokiniana* beads in the first 5 days which reached the maximum of 99.9% within 16 days. Comparing the nitrate removal rate of samples containing different ratio of algae to water indicates that using higher volumetric ratio than 12.5% did not result in improved performance. Therefore, the ratio of 12.5% was used in the batch reactors on the remaining experiments. Macro- and micro- nutrients required for growth were introduced to the solution





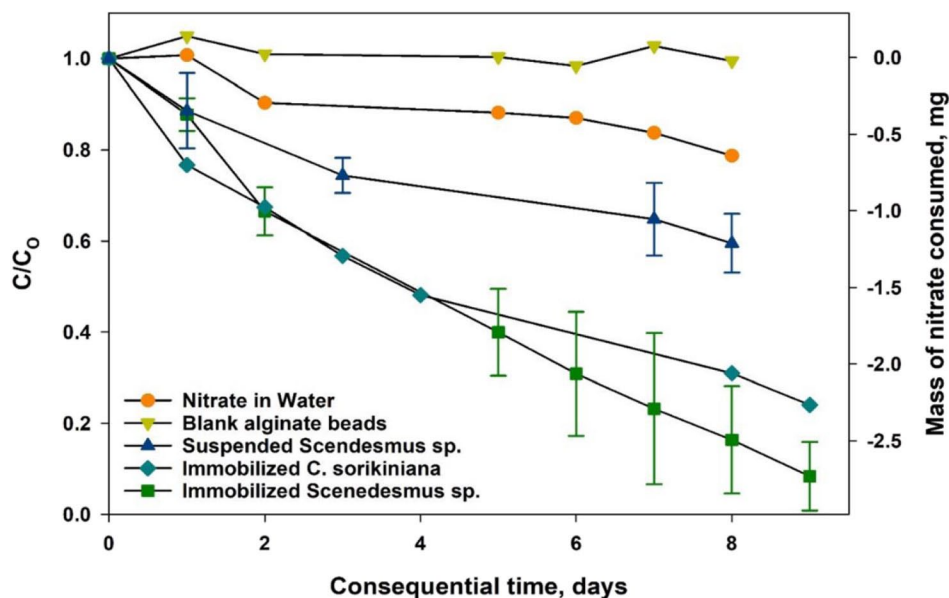
**Fig. 2** Effect of four different ratio of immobilized *C. sorokiniana* cells to water: **a)** percentage of nitrate present in the solution over time, **b)** the average pH, DO, and conductivity, and **c)** change in COD concentration of the bulk solution.

only through the added bead volume. Since nitrate removal pattern is equal beyond 12.5%, this was an indication that this ratio provided sufficient nutrients. The average removal rate for the reactor containing 12.5% algal beads was 0.190 mg N/d.

Figure 2b shows the average values of pH, DO, and conductivity in the bulk solution for each reactor during 16 days of growth. There was a direct relationship between the volume of algal beads in the reactors and the pH of the bulk solution. This is explained by photosynthesis occurring in presence of increased algal cell numbers. The same trend occurred for DO. Since the algal cells had access to both organic carbon source (glucose) and inorganic carbon source (carbon dioxide from the air) and they were kept in presence of light, we surmise that they switched between autotrophic and heterotrophic growth, depending on the energy source (light or glucose) availability. The concentration of glucose used in the growth study (150 mg/L) was substantially lower than what was suggested by other literatures (5–80 g/L) (Samejima and Myers 1958; Shi et al. 1999); therefore, it is possible that the glucose initiated the heterotrophic growth but was the limiting factor for the long-term heterotrophic growth.

Moreover, there is a direct correlation between the volume of algal beads and conductivity of the bulk solution. The algal beads were kept in  $\text{CaCl}_2$  solution overnight to solidify before application. By comparing the conductivity of the control treatment (with no microalgae or alginate) and the other reactors, it is evident that the high value of conductivity is mostly because of the presence of calcium and chloride ions. The average concentration of chloride in the batch reactor with 12.5% of algal beads was estimated to be  $89 \pm 2.2$  mg/L.

Figure 2c compares the COD consumption vs. time for three of the reactors. The reactors containing 6.25% and 12.5% algal beads follow a very similar trend in glucose consumption. In the first 48 h, about 35% and 45% of the glucose was consumed by algal cells in the reactors with 6.25% and 12.5% algal beads, respectively. The reactor with 25% algal beads, however, had a sharp reduction on day 2. About 65% of the glucose was consumed at the first 48 h by algal cells in this reactor. At the last 4 days (days 5–9), the glucose concentration stayed almost constant indicating algae switching from heterotrophic to autotrophic mode.



**Fig. 3** Nitrate concentrations present in the solution over time in presence of immobilized *C. sorokiniana*, *S. sp.* beads, and suspended *S. sp.*

### 3.3 *Chlorella sorokiniana* vs. *Scenedesmus* species: Effect of Starvation and Immobilization

Comparing the strains of *C. sorokiniana* and *S. sp.* indicates that immobilized *S. sp.* were slightly better than *C. sorokiniana* in nitrate removal (**Fig. 3**). Consequently, *S. sp.* were further used in the sequencing batch reactor evaluation. *S. sp.* beads removed 60% of  $\text{NO}_3$  after 5 days and 90% of  $\text{NO}_3$  was eliminated in 9 days. For *C. sorokiniana*, the removal rate was 58% in 5 days and 78% in 9 days.

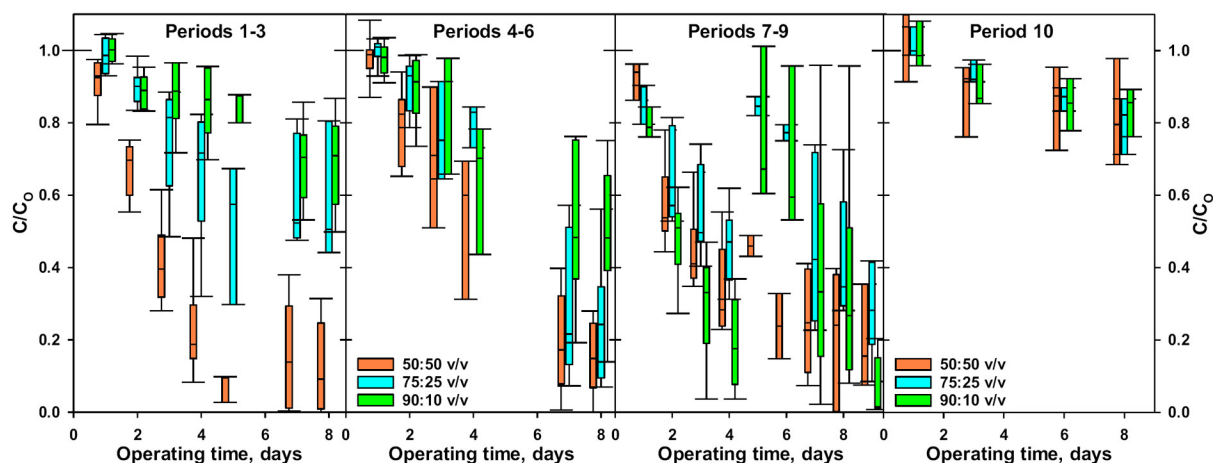
Moreover, the effect of starvation on nutrient removal was evaluated. *S. sp.* beads were stored without nutrients for 6 days until their utilization in bioreactors. While there was an increase in DO concentration and pH levels for both starved and non-starved *S. sp.* cells, the rate of increase (in both DO and pH) was higher in non-starved cells, which was an indicator of increased levels of photosynthesis occurred in cells without any starvation period. Between the biological compounds, RNA, DNA, and protein are indicators of physiological status due to their direct relationship to cell division and growth. Positive correlations between the ratio of RNA/DNA and growth rate have been obtained in a variety of organisms, such as microbial communities

and natural and synthesized phytoplankton populations (Berdalet et al. 1994). *S. sp.* is one of the microalgae species with the ability to produce and accumulate triglyceride (TAG) under nitrogen starvation conditions (Breuer et al. 2012). Under limited nitrogen conditions, the rate of DNA, RNA, and chlorophyll production is impaired. As a result, less electrons are generated by photosynthesis which will mostly be used to produce TAG (Breuer et al. 2013). As shown in Fig. S1, the starvation did not improve the nitrate removal rate. N-starvation was studied in *Heterocapsa sp.*, grown in batch cultures, and it was reported that RNA was markedly impacted by N-starvation cells in the intermediate and stationary phases. The N-starved treatments also resulted in a remarkable decrease in the net photosynthetic rate and chlorophyll content per cell which could be the main reason of deterring performance of the starved immobilized cells (Berdalet et al. 1994).

Nitrate removal by suspended algal culture was compared to immobilized cultures. The same mass of algae was used in both conditions. The nitrate removal rate for suspended and immobilized culture was the same at day 1 as seen in Fig. 3. In the next 7 days, however, the nitrate uptake by immobilized cells was significantly higher than it was by the suspended culture. Suspended *S. sp.* cells were able to remove 58% of nitrate in 8 days whereas the immobilized beads reached 80% removal rate during the same time. Higher rate of nitrate removal by immobilized cells could be explained by higher light intensity reaching the algal cell. For suspended cultures, as biomass density increases, the light penetration depth will decrease. This will result in smaller percentage of algal cells being effectively illuminated (J. F. Wang et al. 2015). An overall light penetration throughout the reactor was estimated for both immobilized and suspended cultures. For the immobilized cells at day 0, 44% of the light penetrated the entire diameter of the photobioreactor vs. 42% measured for the suspended cells. On day 8, these values changed to 35% and 20% for immobilized cells and suspended cultures, respectively. Light intensity and distribution inside the immobilized cells were not quantified.

### **3.4 Nitrate Removal in a Sequencing Batch Reactor**

While nitrate was successfully removed in batch reactors, it was essential to know how many cycles, the beads could be reused for water



**Fig. 4** Nitrate removal in a sequencing batch reactor operated with different volumetric ratio of alginate to algae. Box plots summarize several periods for data clarity.

treatment. Therefore, the bead lifetime was assessed using sequencing batch reactors. Every 9 days, the treated water in each reactor was discarded and the reactors were filled back with 400 ml nitrate-contaminated water matrix and 125 mg/L glucose. Besides the nutrients embedded within the beads during manufacturing, no additional nutrients were added throughout these experiments. Additionally, four sets of experiments with different ratios of algae to alginate were used: 75:25, 50:50, 25:75, and 10:90 (v:v %), respectively. Different ratios were used to assess the minimum number of initial algae needed without compromising the performance. Maintaining high nitrate removal performance with smaller algae concentration results in reduced cost for the required initial algae cultivation. **Figure 4** shows the removal efficiency and the performance of the immobilized *S. sp.* beads over their lifetime until they failed to remove additional nitrate. The ratio of 75:25 (algae: alginate) was discontinued as alginate was not sufficient to support the bead structurally. The higher initial algae concentration within the beads (50:50) showed the maximum removal efficiency at the first 75 days of operation (periods 1–7). The performance obtained by the 25:75 ratio kept improving over time. The performance is summarized in Fig. 4 by dividing the data in three distinct periods of operation. By period 4, the performance of 25:75 ratio was almost equal to the 50:50 ratio. Similar performance between both ratios was maintained throughout period 6 but 25:75 ratio deteriorated

without a known reason thereafter. The 10:90 ratio, which has initially the lowest algae concentration, took longer time to catch up to the same performance as 50:50 ratio. By period 6, the performance of 10:90 ratio was comparable to 50:50 ratio and surpassed it by period 8. At period 10, all the beads showed a deteriorating performance.

The initial algal density has a major impact on nutrient removal from water. The higher algal density within the beads causes more nutrients as well as glucose assimilation. The initial average algal density for the beads with 50:50, 25:75, and 10:90 ratios were 8.14, 7.04, and 6.42 mg/L, respectively. Toward the end of the treatment, nitrate removal efficiency of the 50:50 ratio beads was lower than that of the 10:90 ratio (Fig. 4). It was observed that the dense algal mass in the concentrated culture were metabolically less active toward the end of the treatment process. The high algal density causes self-shading, an accumulation of auto-inhibitors, which reduces photosynthetic efficiency (Xu et al. 2015). These results suggest that having a concentrated culture can shorten the retention time for nitrate removal and algal cells can be replaced before self-shading substances accumulated to a toxic level.

**Table 1** shows the concentration of metals in the water matrix upon the preparation of the beads (period 1) and at the end of the treatment process (period 10). As it's shown, Na, Mg, P, K, Ca, Zn, and Cu were the dominant elements in the water. The nutrients that were added to the algae solution prior to making the beads include all these elements. The more algae content in the beads means more nutrients and that's why the highest metal concentration is observed in water containing 50:50 ratio beads. Most of these elements were partially removed by algal beads at period 10. The only exception was Fe, which was not detectable at period 1, but was diffused from the beads to the bulk solution at period 10. There are also trace amounts of Co, As, Se, and Cd in period 1 which were all removed at the end of the treatment process (period 10).

### **3.5 Algal Growth, pH, DO, and Reaction Rates within Beads**

Algal density of immobilized *S. sp.* in sequencing batch mode was measured periodically using dry cell weight (DCW). Figure S2 shows the change within the first two periods. The density of the algal cells is

**Table 1** The concentration of metals in water matrix used in sequencing batch reactors at periods 1 and 10

Period 1	50:50	62.64±6.90	3.52±0.57	0.27±0.07	78.93±0.05	43.49±0.20	ND	6.87±0.06	25.90±13.32	494.10±28.64	108.60±52.93	1629.73±87.39	191.83±5.94
	75:25	57.39±0.85	0.12±0.06	0.12±0.01	40.95±0.03	32.64±6.40	ND	5.30±0.61	10.53±1.17	419.40±26.60	104.23±26.36	1770.70±34.66	229.90±37.22
	90:10	52.50±0.88	0.06±0.01	0.05±0.02	39.36±0.07	21.90±1.12	ND	3.67±0.15	ND	ND	115.27±23.64	3178.33±23.46	250.13±11.82
Period 10	50:50	4.34±0.32	ND	0.09±0.01	52.29±4.65	6.02±1.22	17.59±1.44	ND	8.96±0.14	ND	ND	ND	ND
	75:25	4.83±0.12	0.03±0.01	0.06±0.02	39.66±9.87	14.17±0.12	40.09±2.34	ND	10.57±0.76	ND	ND	ND	ND
	90:10	4.56±0.65	0.17±0.04	0.06±0.02	50.32±7.23	16.89±6.72	45.60±10.55	ND	8.05±1.33	ND	ND	ND	ND

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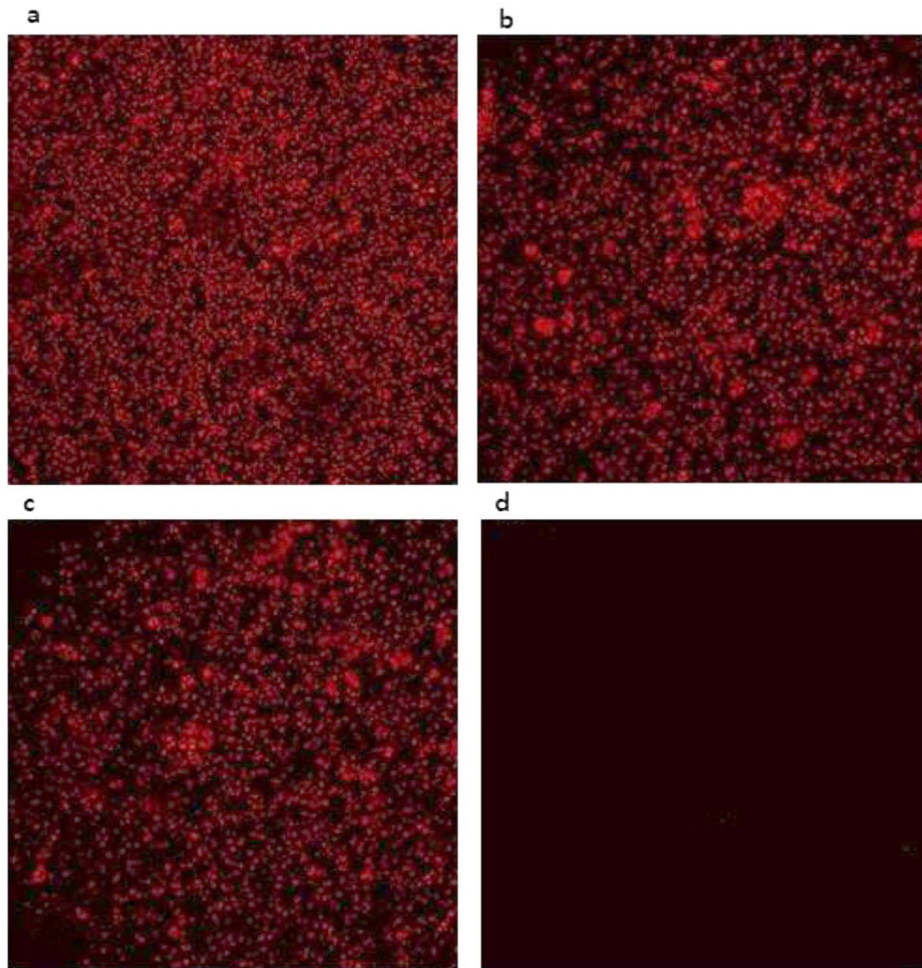


gradually increasing over time at the first period (days 0–9). The increase of cells density within the beads indicates that algae can still perform photosynthesis after immobilization, which agrees with previous reports of *C. vulgaris* immobilized in alginate (Ruiz-Marin et al. 2010). During the second period (days 9–17), however, the algal density increased slightly until day 14 after which it remained mostly constant. This indicates that the stationary phase of growth was reached. The slight algal density decrease could be explained by insufficient nutrients in the bulk solution. At the end of every period of sequencing batch operation, treated water was discarded and the beads were washed out. During this process, some nutrients that diffused from the beads to the bulk solution were washed out and never recovered.

Figure S3 shows the changes in pH and DO of the bulk solution within selected periods of growth studies in sequencing batch mode. As shown, there was a large drop in the concentration of DO every week. The color of the beads turned from green at period 1 to white at period 10. When algal cells photosynthesize, they use carbon source and produce oxygen and increase the concentration of DO in the solution. Photosynthesis also increases the pH of water as it removes carbon dioxide. When photosynthesis slows down or stops, both DO and pH tend to decrease. Therefore, the drop in the DO and pH, and change of color are all indicators that photosynthetic rate is decreasing in the beads which could be due to nutrient depletion, as it was explained in Section 3.4. The nitrate uptake in each sequencing batch reactor was simulated using the first order reaction kinetics ( $\ln C/C_0 = -kt$ ). The  $k$  value was maximum for the samples with 50:50 ratio beads (0.18 l/d) which was an indicator of faster nitrate removal rate by them. For the samples with 25:75 and 10:90 ratio beads, the  $k$  value was 0.10 and 0.07 respectively (Fig. S4).

### 3.6 Viability of Algal Cells Within the Beads

**Figure 5** shows optical images of *S. sp.* cells immobilized in sodium alginate (a) initially, (b) after 10 days, (c) after 20 days, and (d) after 112 days from the start of experiments in sequencing batch reactor. The red color is an indicator of healthy algal cells. The area covered with live cells was 104,385 pixels initially, 104,411 pixels after 10 days of growth, 75,702 pixels after 20 days of growth, and 0 pixels

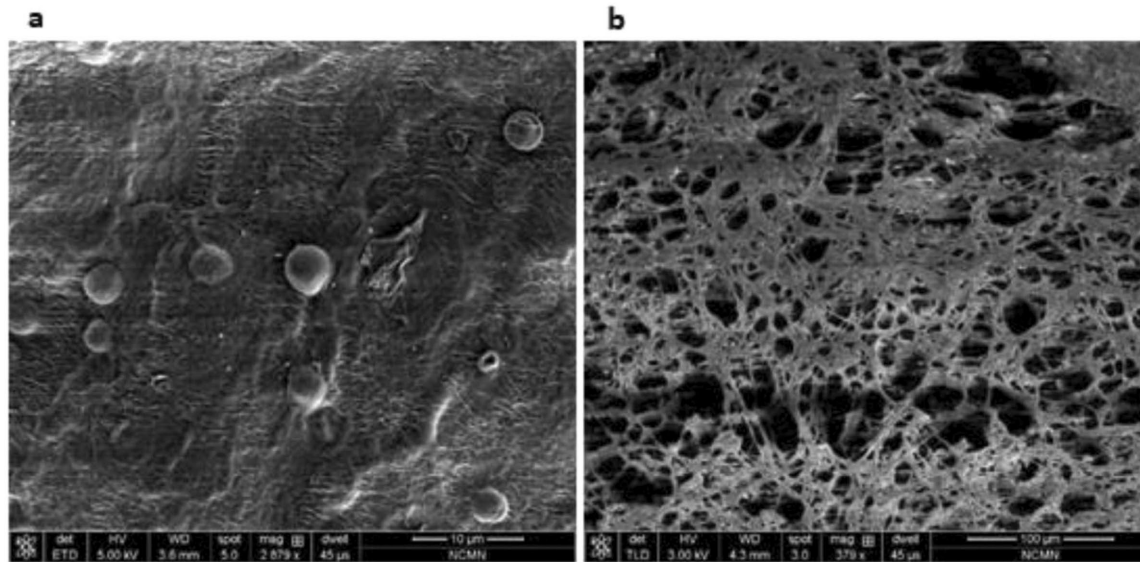


**Fig. 5** Optical images of immobilized *Scenedesmus* cells **a)** initially, **b)** after 10 days, **c)** after 20 days, and **d)** after 112 days from the start of experiments in sequencing batch mode.

after 112 days. The results from microscopic images match the algal density results measured by means of DCW (Section 3.5).

Detailed images of the algae within the bead are shown through a scanning electron microscope (SEM) in **Fig. 6**. As shown in Fig. 6a, sodium alginate matrix is an effective support matrix for immobilizing *S. sp.* cells, which have the diameter of 3–4  $\mu\text{m}$ . This porous structure of sodium alginate also allows the diffusion of nutrients between the bulk liquid and the algae, which allows photosynthesis of algal cells.

After 112 days of operation, however, sodium alginate matrix lost its firm structure due to the loss of the calcium ions in the solution in sequencing batch mode. Calcium ions provide cationic bridges between

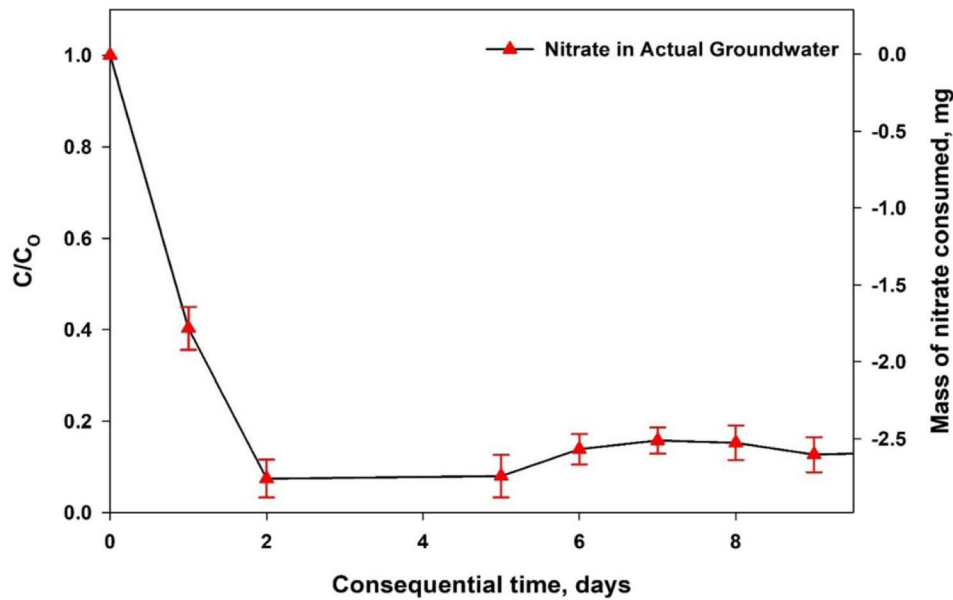


**Fig. 6** SEM images of immobilized *S. sp.* cells on the surface of sodium alginate at day 0 (**a**) and day 112 (**b**) of operation.

the guluronic-rich regions along the biopolymer backbone (Kleinübing et al. 2013) and its depletion damages the structure of the beads. At day 112, no algal cells were observed in sodium alginate matrix and the beads were surrounded with strains of fungi. As shown in Fig. 6b, the alginate mat became very porous with the diameter of 13–20 nm which allowed algal cells to escape from the matrix. Fungus are ubiquitous in the environment and the media is a great environment for them to quickly colonize and flourish. In our case, open bottles were used as reactors for 112 days which could cause fungus contamination in long term.

### 3.7 The Application of Actual Groundwater

Immobilized *S. sp.* were tested using actual groundwater that was collected from wells in Hastings, NE. Hastings was chosen because it is experiencing nitrate contamination in its water supply aquifer. About 12% of the municipal wells in Hastings were reported to have nitrate concentration above MCL in 2016. High levels of nitrates migrating through the aquifer and into the city wells are a potential health risk for people in this town. The estimated cost of installing a traditional



**Fig. 7** Percentage of nitrate present and mass of nitrate consumed over time in actual groundwater.

water treatment facility to eliminate nitrate contamination is \$75 million. Currently, the city is working closely with water quality experts to find a sustainable and efficient solution to reduce the high level of nitrates (City of Hastings 2016).

Upon collection, the concentration of nitrogen, phosphorous, and COD in the groundwater sample was measured and found to be 8.9, 2.8, and 100 mg/L, respectively. As shown in **Fig. 7**, 90% of nitrate was eliminated within 2 days without adding any external carbon source. The groundwater was rich in nutrients described above, which provided a more suitable environment for algal cells to grow without needing any additional nutrients/carbon source.

#### 4 Conclusions

Immobilized *S. sp.* beads were more efficient in removing nitrate from water than the suspended cell system and can be used up to 100 days in sequencing batch mode. Heterotrophic cultivation of immobilized *S. sp.* is an efficient biological method for nitrate removal as it facilitates large volume applications with production of valuable biomass

which is easy to harvest. In natural water, no need for additional carbon source is required for successful removal. Actual groundwater is more suitable for nitrate removal than simulated lab water. Algae migration from the immobilized phase to the bulk water is minimal.

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**Conflict of Interest** The authors declare that they have no conflict of interest.

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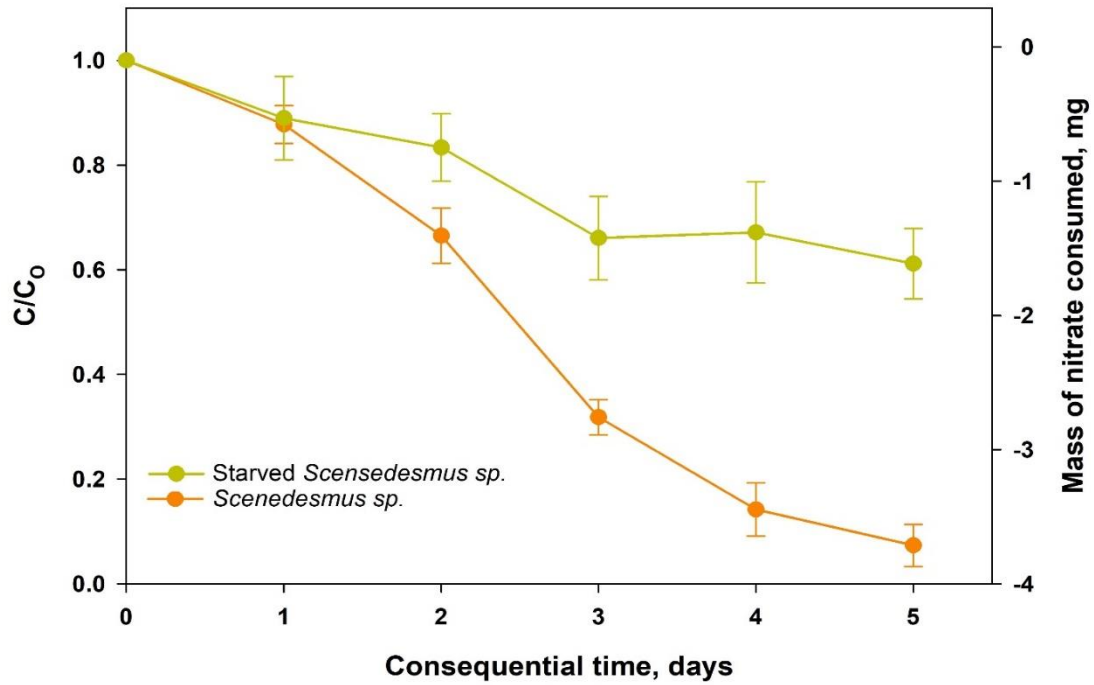
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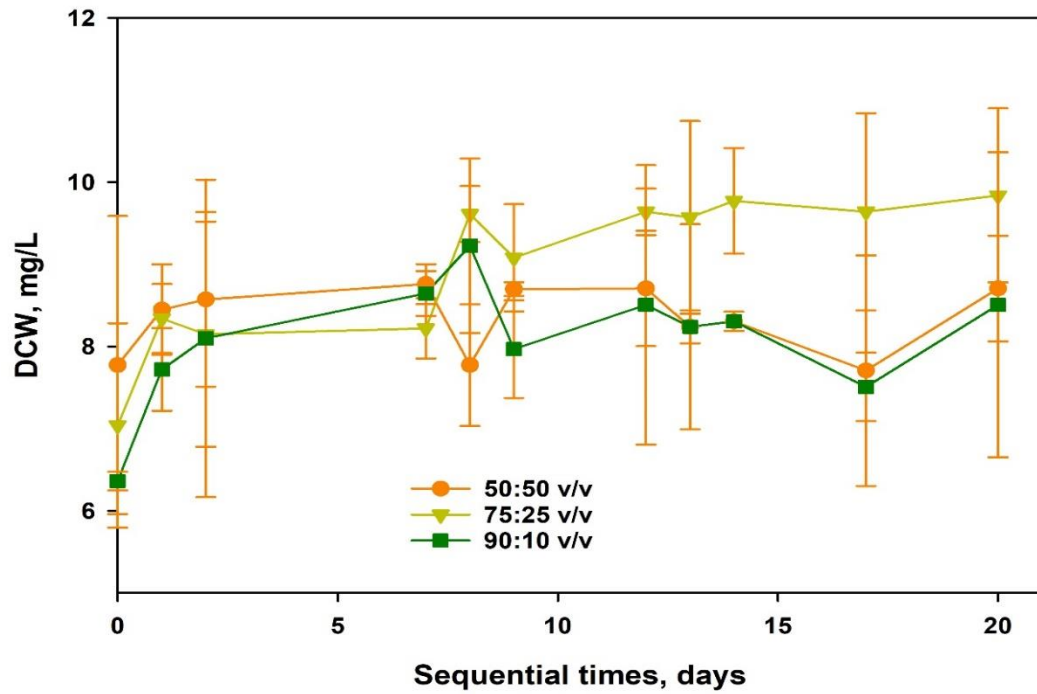


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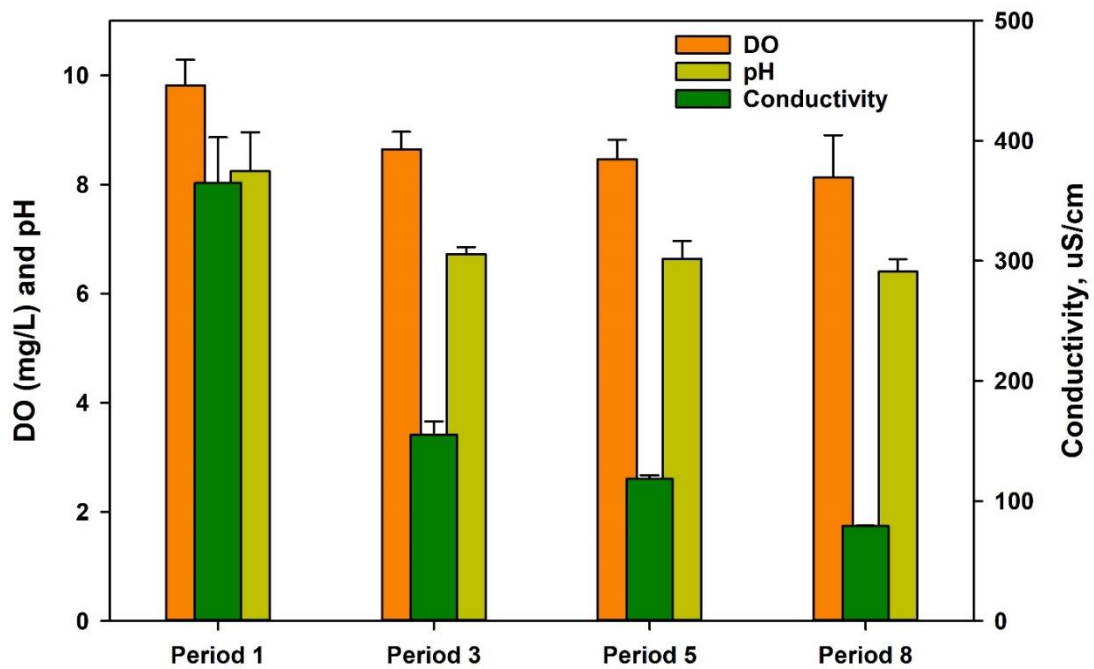
## Supplemental materials



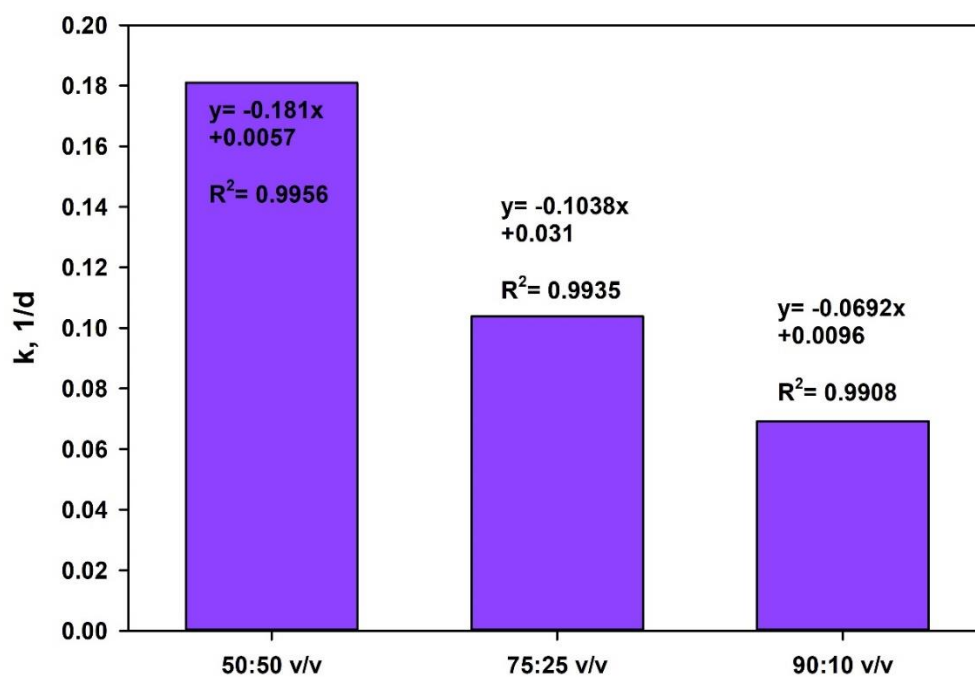
*Fig S1- The impact of starvation on nitrate uptake rate by immobilized S.sp beads*



*Fig S2-DCW of the algae in the beads with different volumetric ratio of alginate: algae, used in a sequence batch reactor*



**Fig S3-** Average pH, DO and conductivity of the bulk solution over the 8 periods of the experiments in sequencing batch mode



**Fig S4-** Average  $k$  value calculated for each reactor in a sequence batch reactor